American Journal of Hematology 58:224–230 (1998)

Increased Tyrosine Phosphorylation of Band 3 in Hemoglobinopathies

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In order to investigate the tyrosine phosphorylation of band 3, we performed immunoblotting of intact red cells using anti-phosphotyrosine antibody of 21 patients with sickle cell disorders (11 SS, 5 Sβ, 5 SC), 7 patients with β thalassemias (5 β thal intermedia, 2 δβ thal), 10 normal controls, and 1 patient with hereditary spherocytosis. They had not received transfusion for the last 4 months and all were clinically stable. Our results showed an increased tyrosine phosphorylation of two proteins, in the 100 and 80 kD regions, in sickle cell and β-thalassemic red cells when compared to the normal controls and to the patient with hereditary spherocytosis. Immunoprecipitation of the lysed red cells with anti-band 3 antibody and immunoblotting with anti-phosphotyrosine antibody confirmed that the 100 kD tyrosine phosphorylated protein was band 3. In the sickle cell disease group, the band 3 tyrosine phosphorylation varied from 2- to 10-fold increase compared to control (x ± SD; SS = 7.8- ± 2.7-fold; SC = 3.8- ± 1.3-fold; Sβ = 5.2- ± 2.0-fold). It was also higher in the β-thalassemic group (β-thal = 4.3- ± 3.7-fold). There was no significant difference in tyrosine phosphorylation among the various groups tested, except when we compared the phosphorylation in intact red cells of patients with sickle cell anemia and hemoglobinopathy SC (U = 6, *P* < 0.02). The tyrosine phosphorylation of band 3 was increased in hemoglobinopathies even in the absence of high reticulocyte count. At least two mechanisms might be involved in the increased tyrosine phosphorylation of band 3 in these hemoglobin disorders, probably related to the endogenous reactive oxygen intermediates generated by the abnormal erythrocyte: an inhibition of protein tyrosine phosphatase activity or an activation of the protein tyrosine kinase p72syk. Am. J. Hematol. 58:224–230, 1998.

Key words: band 3; tyrosine phosphorylation; hemoglobinopathy; sickle cell; beta-thalassemia; red cell membrane

INTRODUCTION

Many protein functions are controlled by reversible side-chain modifications as their state of phosphorylation. Protein phosphorylation on tyrosine residues has been implicated in the regulation of normal cell growth as well as in the onset of cell transformation. One of the most intriguing aspects of the cellular protein tyrosine kinases (PTKs) is their presence in terminally differentiated cells lacking nucleus and undergoing neither division nor differentiation as the erythrocytes. Human erythrocytes contain a membrane bound tyrosine kinase that is apparently specific for the transmembrane anion transporter, band 3 [1]. The tyrosine phosphorylation site has been identified preferentially on tyrosine 8, at the N-terminus of band 3 [2,3]. This region of the cytoplasmic domain of band 3 has high affinity binding sites for several peripheral proteins including hemoglobin [4,5], hemichromes [6], and glycolytic enzymes [7–9]. Although there is no evidence for an effect of band 3 tyro-

Contract grant sponsor: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP); Contract grant sponsor: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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Received for publication 30 June 1997; Accepted 18 March 1998

TABLE I. Hematological Data of Patients With Hemoglobinopathies (Mean ± SD)*

Diagnosis	Age (years)	Hb (g/dl)	MCH (pg)	MCV (fl)	HbA2 (%)	HbF (%)
SS (n = 11)	25.0 ± 6.1	8.4 ± 1.5	30.8 ± 1.9	94.1 ± 7.7	2.3 ± 0.60	7.0 ± 4.4
Sβ (n = 5)	26.6 ± 9.4	8.7 ± 1.2	24.9 ± 6.5	66.2 ± 8.9	3.9 ± 1.0	7.4 ± 6.7
SC (n = 5)	34.0 ± 10.7	11.4 ± 2.0	28.5 ± 2.2	85.8 ± 5.5	—	1.5 ± 0.7
βthal (n = 7)[a]	30.6 ± 9.0	7.5 ± 1.2	23.7 ± 1.7	70.6 ± 3.06	3.5 ± 2.1	57.01 ± 22
HS (n = 1)	58	8.8	32	94	2.3	1.3

*Hb = hemoglobin; MCH = mean corpuscular hemoglobin; MCV = mean corpuscular volume; SS = sickle cell anemia; Sβ = Sβ thalassemia; SC = hemoglobinopathy SC; βthal = βthalassemia; HS = hereditary spherocytosis.
[a]Includes 2 patients with δβ-thalassemia.

sine phosphorylation on its function as a membrane skeletal anchoring site [10], the phosphorylation of the purified cytoplasmic domain of human erythrocyte band 3 by a protein-tyrosine kinase (PTK) results in the inhibition of binding of aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphofructokinase, and hemoglobin [11]. Thus, it is possible that the PTK in human erythrocyte regulate glycolysis through the Tyr phosphorylation of band 3.

On the other hand, a mutant hemoglobin might interact abnormally with the membrane. For example, while Hb A shows reversible binding to ghosts, Hb S shows an additional irreversible component [12]. Also denatured hemoglobin, in the forms of Heinz bodies or hemichrome, binds more readily than Hb A to band 3 on the inner membrane surface [6,12–14] and causes widespread redistribution of skeletal proteins [15]. Sickle erythrocytes have an abundance of denatured sickle hemoglobin in the form of micro Heinz bodies associated with overlying clustered band 3, ankyrin, and glycophorin [16,17] and an increased deposition of immunoglobulin [16,18,19]. However, the relationship between this abnormal binding and the band 3 tyrosine phosphorylation is unknown. Numerous studies of in vitro phosphorylation in ghosts or intact cells have suggested that sickle cell erythrocyte membranes have an abnormal level of protein phosphorylation compared with normal membranes [10,20–23]. Spectrin phosphorylation is reduced, whereas phosphorylation in band 3 and in the region of bands 4.5–4.9 is increased. In this study we report that, in sickle cell and β thalassemic erythrocytes, the band 3 tyrosine phosphorylation is higher than in normal erythrocytes.

MATERIALS AND METHODS

Patients

Twenty-eight patients with hereditary hemoglobinopathies (11 SS, 5 Sβ, 5 SC, 5 β thal intermedia, 2 δβ thal) were studied. They had not received transfusion for the last 4 months. All were clinically stable and the diagnosis was based on clinical, familial, and laboratory data, including electrophoresis on cellulose acetate at pH 8.9 and on agar gel at pH 6.2, solubility test, estimation of HbF and HbA2 [24], and, in most cases, family studies. All gave informed consent and the study was approved by the Ethical Committee of this hospital. Ten normal individuals and 1 patient with hereditary spherocytosis were used as controls. Clinical and hematological data of these patients are presented in Table I.

Materials

Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), protein assay kit, immunoblotting apparatus, and nitro-cellulose were from Bio-Rad (Richmond, CA). Phenylmethylsulfonylfluoride (PMSF) and DTT were from Sigma Chemical Co. (St. Louis, MO). Percoll and protein A-sepharose were from Pharmacia (Uppsala, Sweden). ^{125}I-Protein A was from ICN Biomedical (Costa Mesa, CA). Monoclonal antiphosphotyrosine antibodies were from Santa Cruz (Santa Cruz, CA).

Methods

Tyrosine phosphorylation of red cell was studied by immunoblotting as previously described [25] with minor modification.

Intact red cell tyrosine phosphorylation. Five milliliters of fresh blood were collected from patients and from normal controls randomly, using EDTA as anticoagulant and 1 mM of sodium vanadate and immediately kept on ice. In this way, the control's samples were collected sometimes at the beginning, middle, or end of the entire sample collection. For each experiment, the timing for collection of all samples (usually 2 controls and 5 patients) varied from 20 to 30 min. After the last sample was collected, we immediately started the experiments as follows: the red cells were washed 3 times in ice cold PBS with 1 mM of sodium vanadate. The washed erythrocytes at 50% of hematocrit were boiled for 4 min in 5 vol of Laemmli sample buffer with 100 mM DTT and 40 μl of this mixture was resolved on 8% SDS-PAGE.

In order to study the band 3 phosphorylation without interference of sodium vanadate we also performed experiments on total blood collected in EDTA and immediately boiled in Laemmli sample (0.1% bromophenol

blue; 1M sodium phosphate, pH 7.0; glycerol 50%; SDS 10%; 0.2 mM DTT) as described above.

Molecular weight standards were myosin (205 kDa), βgalactosidase (116 kDa), bovine serum albumin (80 kDa), and ovalbumin (49.5 kDa).

Electrotransfer of proteins from the gel to nitrocellulose was performed for 2 hr at 100 V (constant). Non-specific protein binding to the nitro-cellulose was reduced by preincubating the filter overnight at 4°C in blocking buffer (3% non-fat dry milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20). The nitro-cellulose blot was incubated with anti-phosphotyrosine antibodies or with anti-band 3 antibodies diluted in blocking buffer for 18 hr at 4°C and washed for 60 min with the blocking buffer without BSA. The blots were incubated with 2 μCi of ^{125}I-protein A (30 μCi/μg) in 10 ml of blocking buffer for 1 hr at 22°C and then washed again as described above for 2 hr. ^{125}I-protein A bound to the anti-phosphotyrosine and anti-band 3 antibodies was detected by autoradiography using preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens at-70°C for 12–48 hr. Band intensities were quantitated by optical densitometry (Hoefer Scientific Instruments, San Francisco, CA; model GS 300) of the developed autoradiogram and the area under the peaks was determined by the computer program GS 365 W, version 3.01. In all the experiments we ran in parallel at least two controls in order to obtain a ratio of phosphorylation of patient and control. The results were expressed as a ratio to controls (P/C) based on the following calculation:

$$\frac{\dfrac{\text{Phosphorylated Band 3 of Patient}}{\text{Total Band 3 of Patient}}}{\dfrac{\text{Phosphorylated Band 3 of Control}}{\text{Total Band 3 of Control}}}.$$

Isolation of erythrocytes depleted of reticulocytes by Percoll. Blood (20 mL) was collected in EDTA and 1 mM of sodium orthovanadate and immediately kept on ice. The erythrocytes were washed in PBS and 5 ml of the washed erythrocytes were applied on the surface of 20 ml 75% Percoll (75 mL Percoll net, 25 mL PBS-4x and 1 mM sodium orthovanadate). Then they were centrifuged at 45,000*g* for 30 min at 4°C. After the run, 1 ml fractions were collected from the top and from the bottom of the gradient. The cells were washed once in PBS, solubilized in Laemmli sample buffer with 100 mM DTT, and 50 μL of this mixture was resolved by SDS-PAGE.

Immunoprecipitation. Ghost (500 μL) was resuspended in 500 μL of buffer A (0.1 mol/L Tris-HCl, pH 8.5, 0.15 mol/L NaCl, 5 mmol/L EDTA, 0.5% NP-40) containing 1% sodium dodecyl sulphate (SDS) and 1 mM sodium orthovanadate, placed in a boiling water bath for 2 min, and then diluted to 5.0 mL with buffer A with sodium orthovanadate. Triton X-100 was added to 5% final concentration. Rabbit antihuman band 3 polyclonal antibody (100 μl) was then added, and the samples incubated overnight at 4°C with gentle shaking. Thereafter, 100 μl of protein A-sepharose was added and the samples incubated for another 4 hr at 4°C with gentle shaking. The protein A-sepharose beads were then collected and washed 1 time with buffer B (1M Tris, 0.2M EDTA, 10% of Triton X-100 0.2M, 2 mM sodium orthovanadate). Beads were removed by centrifugation and the pellet was resuspended in 40 μL SDS sample buffer and boiled for 5 min. Following this step, the sample was applied on SDS-polyacrylamide gels.

TABLE II. Tyrosine Phosphorylation of Band 3 in Intact Red Cells of Patients With Hemoglobinopathies (Mean ± SD)†

Diagnosis	Band3 Tyr phosphorylation (fold increase)
SS (n = 11)	7.8 ± 2.7
Sβ (n = 5)	5.2 ± 2.0
SC (n = 5)	3.8 ± 1.3*
βthal (n = 7)[a]	4.3 ± 3.7
HS (n = 1)	0.8

†SS = sickle cell anemia; Sβ = Sβ thalassemia; SC = hemoglobinopathy SC; βthal = βthalassemia; HS = hereditary spherocytosis.
[a]Includes 2 patients with δβ-thalassemia.
*Significantly different when compared with SS (U = 6, $P < 0.02$, Mann-Whitney U test).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were separated by PAGE (8% polyacrylamide) according to the buffer system of Laemmli [26].

Statistical analysis. To compare the different groups of patients, we used the Mann-Whitney U test. Significance was defined as $P < 0.05$.

RESULTS

In this study we analyzed, by immunoblotting with anti-phosphotyrosine antibodies and in the presence of orthovanadate, the tyrosine phosphorylation in intact red cells of patients with sickle cell anemia, hemoglobinopathy SC, Sβ-thalassemia, and β-thalassemia intermedia. Our results showed an increased tyrosine phosphorylation of two bands. In the 100 and 80 kD regions, in sickle cell and β-thalassemic red cells when compared to the phosphorylation obtained in intact cells of normal controls and in one patient with hereditary spherocytosis (Table II, Figs. 1 and 2). The phosphorylation of band 3 in controls was never higher than in patients. The immunoprecipitation of the lysed red cells with anti-band 3 antibody and immunoblotting with anti-phosphotyrosine antibody confirm that the 100 kD tyrosine phosphorylated protein was band 3 (Fig. 3A).

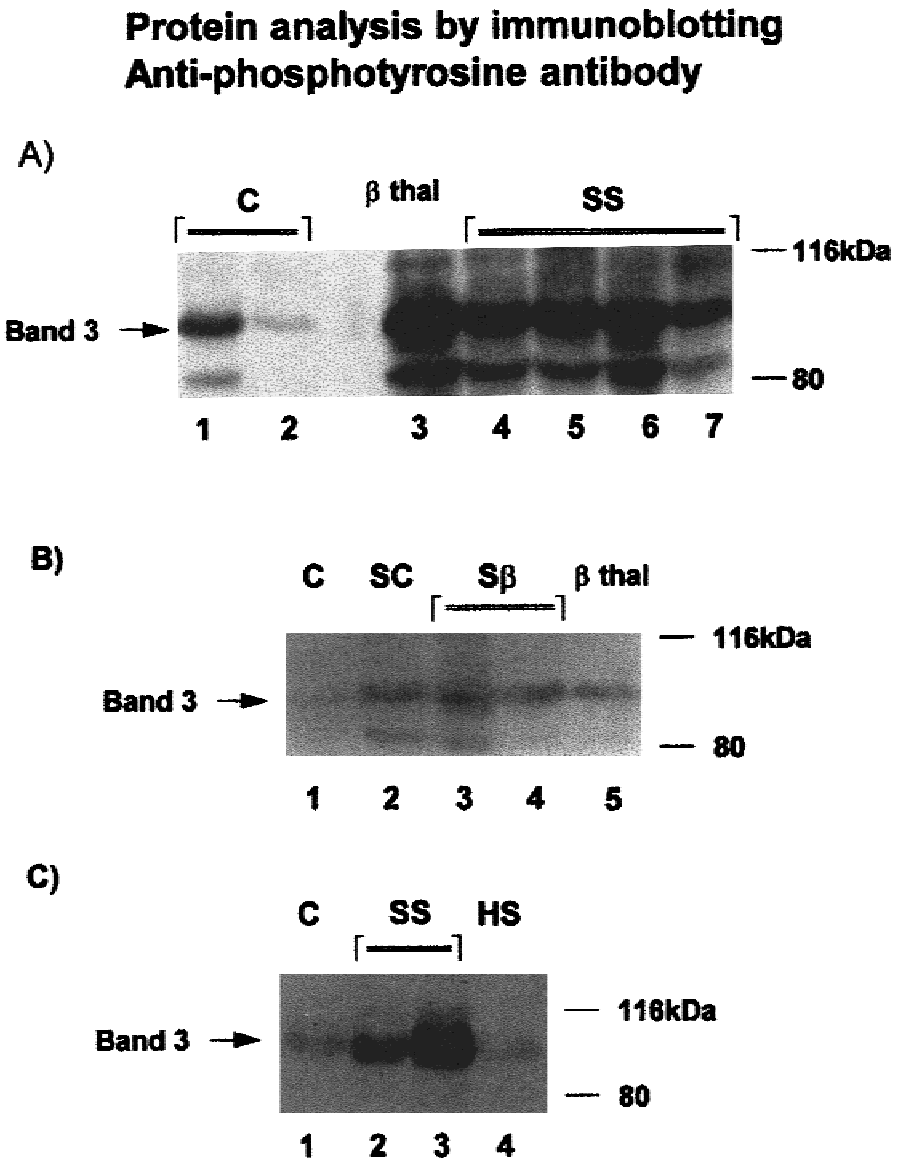

Fig. 1. Tyrosine phosphorylation of band 3 in intact red cells of patients with hemoglobinopathies, analyzed by immunoblotting. A: Lanes 1,2, control; 3, β thalassemia; 4–7, sickle cell anemia. B: Lane 1, control; 2, hemoglobinopathy SC; 3,4, Sβ thalassemia; 5, β thalassemia intermedia. C: Lane 1, control; 2,3, sickle cell anemia; 4, hereditary spherocytosis. The proteins were isolated as described in Materials and Methods. The red cells were washed 3 times in ice cold PBS with 1 mM of sodium vanadate. The washed erythrocytes at 50% of hematocrit were boiled for 4 min in 5 vol of Laemmli sample buffer with 100 mM DTT, and 40 µl of this mixture was resolved on 8% SDS-PAGE and transferred to nitrocellulose. The membrane was immunoblotted with anti-phosphotyrosine antibodies and ^{125}I-protein A, and subjected to autoradiography.

In the sickle cell disease group, the band 3 tyrosine phosphorylation varied from 2- to 10-fold increase compared to control ($x \pm SD$; SS = 7.8 ± 2.7 fold; SC = 3.8 ± 1.3 fold; Sβ = 5.2 ± 2.0 fold). It was also higher in the β-thalassemic group ($x \pm SD$; β-thal = 4.3 ± 3.7 fold).

There was no significant difference in tyrosine phosphorylation among the various groups tested, except when we compared the phosphorylation in intact red cells of patients with sickle cell anemia and hemoglobinopathy SC ($U = 6$, $P < 0.02$).

In order to evaluate the tyrosine phosphorylation without interference of orthovanadate, we also performed experiments without this phosphatase inhibitor. No phosphorylation was observed in the control or sickle red cell.

To ascertain that the tyrosine phosphorylation of band 3 was increased in hemoglobinopathies even in the absence of high reticulocyte count, we separated the young cells on Percoll. The phosphorylation of sickle cells was higher than in the controls, both in the cells obtained from the top and from the bottom fraction of Percoll gradient (Fig. 3B).

DISCUSSION

The characterization of the red cell membrane skeleton gave rise to considerable information regarding skeletal composition, ultrastructure, protein-protein association, membrane anchorage, and molecular defects. Based on this knowledge, special attention has been directed to the study of post-translational or regulatory modifications of membrane. The major erythrocyte membrane proteins are affected by one or several types of post-translational

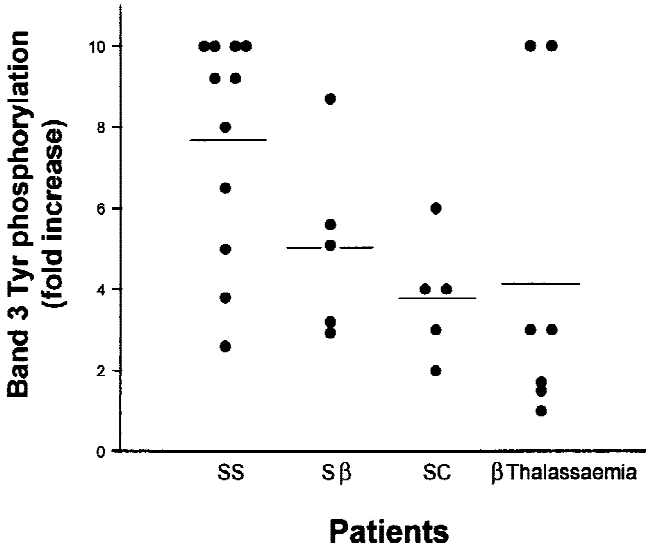

Fig. 2. Band 3 tyrosine phosphorylation in intact red cells of patients with hemoglobinopathies.

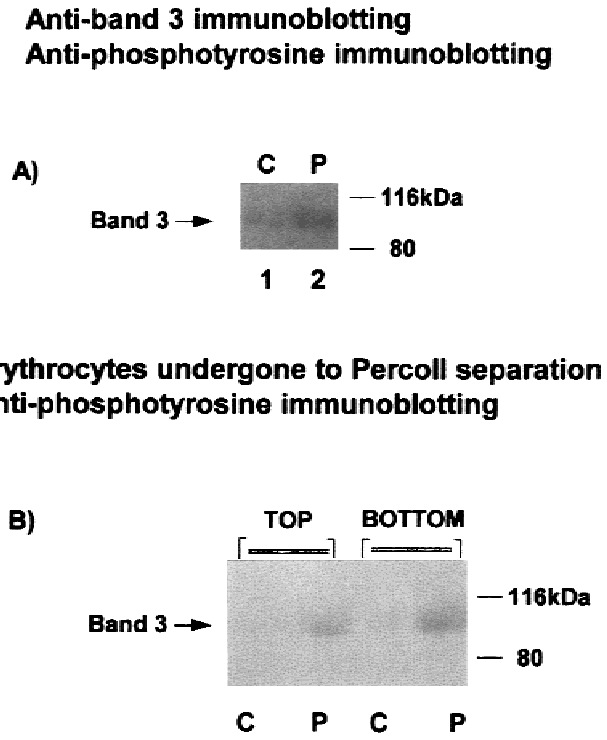

Fig. 3. A: Immunoprecipitation of lysed red cells with anti-band 3 antibody and immunoblotting with anti-phosphotyrosine antibody. C, control; P, sickle cell anemia. The proteins were isolated as described in Materials and Methods, with buffer B and kept on ice; after centrifugation, aliquots from the supernatant were immunoprecipitated with band 3 antibody and immunoblotted with anti-phosphotyrosine antibody and ^{125}I-protein A, and subjected to autoradiography. B: Erythrocytes subjected to Percoll gradient. C, control; P, sickle cell anemia. The erythrocytes were depleted of reticulocytes by Percoll as described in Materials and Methods: 5 ml of washed erythrocytes with PBS were applied on the surface of Percoll 75% and centrifugated at 45,000*g* for 30 min at 4°C. Fractions, top and bottom, were separated by Pasteur pipette and solubilized in Laemmli sample buffer with 100 mM DTT, and 50 µl of this mixture was resolved by 8% SDS-PAGE. Immunoblotting was performed with anti-phosphotyrosine antibody and ^{125}I-protein A, and subjected to autoradiography.

modification, such as oxidation, proteolysis, phosphorylation, and others [10].

In the present study, we demonstrated that band 3 tyrosine phosphorylation is increased in erythrocytes of patients with sickle cell disorders as sickle cell anemia, hemoglobinopathy SC, and Sβ-thalassemia, as well as in β-thalassemia intermedia. Phosphorylation in tyrosine residues is important in the regulation of cell proliferation, differentiation, and metabolism. Very little phosphotyrosine is usually detected in intact cells but a significant increase can be achieved by the use of compounds that inhibit phosphotyrosine phosphatases. The band 3 protein is the main erythrocyte protein that is phosphorylated by tyrosine kinase and this phosphorylation is observed in ghosts and in intact cells when vanadate is present [27]. The kinase apparently associates with the erythrocyte membrane via the acidic N-terminus of band 3 because its association with membranes and phosphorylation of band 3 can be reduced by G3PD, which binds to band 3 in the region of Tyr-8 [1]. Harrison et al. [28] suggested that the protein tyrosine present in erythrocyte might be responsible for regulating glycolysis through the tyrosine phosphorylation of band 3.

Several studies indicate an abnormal phosphorylation of band 3 in sickle cell disease. Initially, the abnormal phosphorylation was seen in isolated ghosts [21] and intact cells incubated with 32P [22]. On the other hand, Johnson et al. [29] showed a normal phosphorylation of spectrin but a substantial increase in 32P in the sialoproteins of sickle erythrocytes.

Recently, Platt and Falcone [30] reported no difference of in vivo tyrosine phosphorylation between normal and sickle cells. The only difference was seen when vanadate was included and peroxide excluded from the incubation mixture. Under these circumstances, as observed in our study, the amount of phosphotyrosine on sickle cells was markedly increased. In fact, a cytosolic phosphatase has been partially purified from erythrocytes and shown to contain phosphotyrosine phosphatase activity against the cytoplasmic domain of band 3 [31,32]. It has been estimated that membrane-associated phosphotyrosine phosphatase activity accounts for 15% of the total cellular phosphotyrosine phosphatase activity [33]. Thus, the increased band 3 phosphorylation is clearly observed when orthovanadate is used. These findings were supported by the recent characterization of a neutral phosphotyrosine phosphatase (PTP) associated with band 3 [34]. This PTP is activated by Mg2+ and inhibited by Mn2+ and vanadate. The behavior of the PTP parallels that of band 3, the major fraction of which is extractable by detergents, with

a minor fraction being anchored to the cytoskeleton. Both band 3 and PTP are co-precipitated when immunoprecipitation is carried out. Thus, it is not surprising that no phosphorylation was observed in the absence of vanadate. This finding, however, does not exclude the possibility that the in vivo tyrosine phosphorylation of band 3 is really increased in sickle cell disease. It is interesting to point out that, different from our findings, Platt and Falcone did not observe any tyrosine phosphorylation in controls, in the presence of vanadate. A possible explanation for this difference is that Platt and Falcone stored the samples for up to 2 days, and we performed our experiments immediately after blood collection. This may have allowed the detection of an in vivo phosphorylation of band 3.

In any study of erythrocytes from patients with anemia, the possible effect of erythrocyte age must be considered. Sickle cells or β-thalassemic cells are young and the observed phosphorylation abnormalities may simply be a property of young erythrocytes. However, our study did not show an abnormal phosphorylation of young normal cells in patients with hereditary spherocytosis and reticulocytosis. Moreover, after Percoll separation of the sickle erythrocyte, the increased phosphorylation was observed in the upper fraction as much as in the lower fraction. Supporting this finding, Johnson et al. [29] reported that the expected age differences between fractions of the sickle cell population are not significant as all cells are relatively young and the phosphorylation in all sickle cell fractions separated on Stractan gradient was similar. These results support the idea that the phosphorylation pattern is characteristic of this disease entity and not simply a consequence of cell age.

A possible explanation for this data is the abnormal oxidant stress in RBC from patients with hemoglobinopathies. Spontaneous autooxidation of normal hemoglobin produces oxygen-free radicals [35], which are neutralized by the normal metabolic antioxidant pathways. Red cells containing some abnormal hemoglobins are under conditions of higher oxidant stress [36]. Mild oxidants were found to stimulate the tyrosine phosphorylation of band 3 [28], possibly by inhibiting a PTP [37] or alterations of redox states [38] preventing the inactivation of vanadate [39]. The effect of hydrogen peroxide on band 3 tyrosine phosphorylation is of interest since in SS cells, the antioxidant defenses are impaired and they spontaneously generate twice the normal amount of activated oxygen species [40]. Membrane-bound p-nitrophenylphosphatase activity has also been described and was observed to be depressed or altered in membranes of individuals with sickle cell disease, congenital hemolytic anemias, and thalassemias [41–43]. Several intracellular changes in thalassemic erythrocytes could account for the oxidative damage in their membrane components since oxidation of excess alpha-hemoglobin subunits and intracellular excess of iron result in increased formation of free oxygen radicals [44].

Another mechanism to explain the increased tyrosine phosphorylation of band 3 in hemoglobin disorders might be related to an activation of the protein-tyrosine kinase p72 syk [38] by endogenous reactive oxygen intermediates as observed in human neutrophils [45].

We also observed, in the patients with hemoglobinopathies, an increased tyrosine phosphorylation of a 80 kD band. This band is probably the protein 4.1 [46], and the characterization of this finding is currently underway in our laboratory.

Although the phosphorylation status of membrane proteins may be critical to the structure and function of the erythrocyte, our understanding of the precise role of phosphorylation is still rudimentary. It is difficult to relate the striking effects of phosphorylation on specific protein-protein interactions measured in vitro with the more complex associations that contribute to membrane properties and skeletal stability in vivo, because there have been no systematic studies relating protein phosphorylation status to those cellular properties that are likely to be affected by phosphorylation. However, reduced ability of sickle protein 3 to bind ankyrin has been described in SS patients [30] and this modification might be related to the abnormal band 3 tyrosine phosphorylation. In addition, the in vitro generation of free radicals induces phosphorylation of band 3 in tyrosine as well as reversible membrane-cytoplasm translocation of glyceraldehyde-3-phosphate dehydrogenase, suggesting that a powerful mechanism of red cell metabolic regulation can modify the energy production during a free radical attack [47].

The data of increased tyrosine phosphorylation of band 3 in hemoglobin disorders strongly suggests that further studies are necessary to elucidate its precise role in the metabolism or membrane skeletal stability of the red cell.

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